

Investigation of the acute toxicity and antimelanoma effects of total leaf extract from *Annona muricata* Linn

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Abstract

Background

Melanoma, the most aggressive and lethal form of skin cancer, accounts for only 4% of all skin malignancies but is responsible for 80% of all skin cancer-related deaths. This study aimed to investigate the safety and antitumor efficacy of the total leaf extract of *Annona muricata* Linn (AME) in a melanoma model in Swiss *albino* mice.

Materials and methods

The safety of AME was evaluated by testing the acute toxicity of AME in healthy mice. Melanoma tumors were induced by subcutaneous implantation of the B16F10 murine melanoma cell line into either immunocompetent or immunodeficient Swiss *albino* mice *via* cyclophosphamide. B16F10-innoculated mice were treated with AME either by oral administration at a dose of 200 mg/kg, by topical application at a concentration of 20 mg/mL or by a combination of oral (100 mg/kg) and topical (10 mg/mL) treatment. Tumor progression was monitored throughout the experiment by measuring tumor size weekly. At the end of the experiment, histological analysis of excised tumor tissues was performed.

Results

The estimated LD₅₀ of AME was approximately 2180.28 ± 84.76 mg/kg. The implantation of B16F10 cells into immunodeficient Swiss *albino* mice resulted in the development of solid tumors the day after implantation. The tumor size increased gradually, reaching its peak at week 3. Histological analysis revealed aggressive invasion of tumor cells into skeletal muscle and neural structures. Compared with no treatment, AME treatment significantly inhibited tumor growth ($p < 0.01$), whereas the combination of oral and topical treatments resulted in the lowest average tumor volume with a moderate invasion profile.

Conclusion

AME was slightly hazardous to Swiss *albino* mice. This extract exhibited antimelanoma effects by inhibiting tumor growth and progression when administered orally, when applied topically, and especially when used in combination. These findings support the potential of AME as a complementary therapeutic agent for melanoma treatment.

1. BACKGROUND

Cancer remains a paramount global health challenge, with 19.3 million new cases and 10.0 million deaths reported worldwide in 2022 [1]. Melanoma, the most aggressive and lethal form of skin cancer,

accounts for only 4% of all skin malignancies but is responsible for 80% of skin cancer-related deaths [2]. Its increasing incidence is correlated with well-established risk factors, including ultraviolet radiation exposure, genetic predispositions, and immunosuppression [3]. Despite therapeutic advances, the prognosis of metastatic melanoma remains poor; the 5-year survival rate for patients with distant metastases is less than 30% [4]. Current treatment modalities include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy [5, 6]. Conventional chemotherapy is often limited by systemic toxicity (such as bone marrow suppression, gastrointestinal toxicity, alopecia, and neuropathies), drug resistance and suboptimal long-term outcomes [7]. These challenges underscore the critical need to explore novel therapeutic agents such as natural plant extracts to increase treatment efficacy and improve patient tolerance [8, 9].

Soursop (*Annona muricata* Linn) has long been used in traditional medicine as a remedy for various ailments. Recent studies have identified more than 120 distinct acetogenin compounds isolated from different parts of the plant, including the leaves, fruits, and seeds. Notably, approximately 60 acetogenins have been found specifically in leaves. These acetogenins are a group of structurally complex compounds that exhibit a wide range of biological activities, including anti-inflammatory, antibacterial, and particularly anticancer properties. Among them, annorecticin and isoannorecticin have been identified as potent compounds that exhibit cytotoxic activity against various cancer cell lines [10]. Given its promising bioactive profile, the development of soursop leaf extract for cancer prevention and supportive therapy holds considerable clinical and practical significance. This study aimed to evaluate the acute toxicity of the total leaf extract of *Annona muricata* and investigate its antitumor effects on Swiss *albino* mice bearing B16F10-induced melanoma tumors.

2. MATERIALS AND METHODS

2.1. Animals: Healthy Swiss *albino* mice, aged 6–7 weeks and weighing 20–30 g, were supplied by the Institute of Drug Quality Control. The animals were housed in same-sex groups of six per cage under controlled environmental conditions (temperature of $22 \pm 2^\circ\text{C}$, relative humidity of $60 \pm 5\%$, and a 12-hour light/dark cycle with lights on from 08:00 to 20:00). All the mice had *ad libitum* access to standard rodent chow and drinking water throughout the study.

All experimental protocols were approved by the Committee on Animal Research and Ethics of the University of Medicine and Pharmacy at Ho Chi Minh City under Decision No. 3674/GCN-HĐĐĐNCTĐV, dated 20/11/2024.

2.2. Total extract from soursop leaves: Soursop leaves were harvested in May 2024, washed, air-dried, and ground into a crude powder with 5–10 mm cubes. The leaf powder was subjected to exhaustive reflux extraction via 96% ethanol. The extraction was performed at atmospheric pressure under reflux conditions at the boiling point of the solvent (three cycles of 3 hours each, with a leaf powder-to-solvent ratio of approximately 1:10 per cycle). The combined extracts were concentrated at 45°C under reduced pressure, yielding *Annona muricata* leaf extract (AME) with an extraction efficiency of approximately 5%

and a humidity of 13%. The resulting ethanol extract was stored under refrigerated conditions for subsequent animal experiments.

2.3. Chemicals: Endoxan (cyclophosphamide 500 mg) was acquired from Baxter Oncology, Germany; 10% formalin was obtained from Xilong, China; and Tween 80 was obtained from GHTech, JHD.

2.4. Evaluation of the acute toxicity of AME

The acute oral toxicity of AME was evaluated via the Behrens method [11]. Healthy mice of both sexes were fasted for 12 h prior to AME administration. The AME was suspended in distilled water containing 1% Tween 80 at different concentrations of extract ranging from 100 mg/mL to 600 mg/mL. The mice were orally administered preprepared AME at a volume of 10 mL/kg body weight, delivering extract doses between 1000 mg/kg and 6000 mg/kg. Following AME administration, the animals were continuously observed for the first 4 hours, and survival rates were recorded over the initial 72-hour period. The mice were subsequently monitored daily for 14 days to assess signs of toxicity, behavioral changes, mortality, and other clinical symptoms. The median lethal dose (LD₅₀) was estimated via the Karber and Behrens method [11]. The resulting LD₅₀ value was classified according to the Globally Harmonized System Globally Harmonized Classification System (GHS) for Acute Toxicity Estimate (ATE) guidelines [12].

2.5. Evaluation of the antimelanoma effects of AME in Swiss *albino* mice

2.5.1. Tumor cell culture and cell preparation for implantation in mice

The B16F10 murine melanoma cell line, which was originally derived from the American Type Culture Collection (ATCC, USA), was kindly provided by the Stem Cell Institute, University of Science, Vietnam National University, Ho Chi Minh City. The cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12) at 37°C in a humidified incubator with 5% CO₂.

On the day of implantation, B16F10 cells were detached from the culture flasks by using a mixture of trypsin-ethylene diamine tetraacetic acid and washed twice with phosphate-buffered saline (PBS). The cells were subsequently counted and resuspended in PBS to achieve the concentrations required for subcutaneous injection. Three cell concentrations were prepared as follows: 5×10^5 cells/mL for the administration of 5×10^4 cells; 5×10^6 cells/mL for the administration of 5×10^5 cells; and 5×10^7 cells/mL for the administration of 5×10^6 cells in a 0.1 mL volume of injection.

2.5.2. Establishment of a B16F10 melanoma tumor model

Prior to B16F10 cell implantation, the dorsal right flank ($\sim 2 \times 2$ cm²) of each mouse was shaved to facilitate subcutaneous administration and tumor monitoring. For immunosuppression, the mice in the designated groups received intramuscular injections of cyclophosphamide at a dose of 180 mg/kg on

days – 5, -3, and – 1 relative to the day of B16F10 cell inoculation. The mice were randomly assigned to two experimental groups, an immunocompetent group and an immunodeficient group, the latter of which was induced by cyclophosphamide administration. In each group, the mice were subcutaneously injected with 0.1 mL of preprepared B16F10 cell suspensions (as described in section 2.5.1) at three densities: 5×10^4 , 5×10^5 and 5×10^6 cells per mouse. Moreover, a normal control group (nontumor-bearing mice), in which the mice received a subcutaneous injection of 0.1 mL of PBS, was also included.

2.5.3. Evaluation of the antimelanoma effects of AME

The mice were subcutaneously injected with the optimal density of B16F10 cells to induce melanoma formation. Beginning the following day, the mice received daily treatments with AME via different administration routes:

- PO200 group: Mice were orally given AME at a dose equivalent to approximately 1/10 of the previously estimated LD₅₀.
- TO20 group: Mice received a topical application of 30 µL/mouse of the same AME solution prepared for oral administration, which was applied to the shaved dorsal area.
- Combination group (PO100 + TO10): Mice were treated with a combined regimen consisting of oral administration of AME at 1/20 of the LD₅₀, along with topical application of the same solution to the shaved area.

Moreover, an untreated control group in which melanoma-bearing mice received distilled water orally at a dose of 10 mL/kg body weight was included.

2.5.4. Evaluation criteria

The experimental mice were weighed twice weekly during the experiment. The survival rate was recorded daily, and the latency period of tumor formation was noted. The tumor dimensions (width and length) were measured via a stereomicroscope connected to NIS-Elements software. The tumor volume (mm³) was calculated according to the formula [13]: $V = \frac{\pi L W^2}{6}$, where V is the tumor volume, L is the length, and W is the width of the tumor.

At the end of the experiment, the mice were sacrificed, and the tumor tissue was harvested and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin wax, sectioned at a thickness of 4 µm, and stained with hematoxylin and eosin (H&E). The stained slides were examined under a light microscope for histopathological analysis at different magnifications. Tumor invasion depth was classified according to Clark levels [14].

2.6. Statistical analysis

The data were processed and analyzed via Minitab 20 software and Microsoft Excel 2016. The results are expressed as the mean ± standard error of the mean (SEM). Statistical analyses, including one-way

ANOVA and the Mann–Whitney test, were performed via Minitab 20 software. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Acute toxicity of AME

Within 15 to 60 min following the oral administration of AME extract, the mice exhibited a progressive decline in physical activity, accompanied by an increased respiratory rate and signs of abdominal contraction. Prior to death, affected mice exhibited convulsions and extension of the hind limbs. In the group receiving high doses of AME (> 3000 mg/kg), the majority of the mice died within two days post-administration. The mice that survived beyond the initial 2-day period gradually recovered and exhibited no abnormal clinical signs during the subsequent 14-day observation period. The mortality rate increased in a dose-dependent manner, as shown in Table 1. The LD_{50} of AME was calculated to be 2180.28 ± 84.76 mg/kg on the basis of the Karber and Behren method. According to the GHS, this LD_{50} value places the extract in Category III (slightly hazardous) for oral exposure, which corresponds to substances with LD_{50} values ranging from 2000 to 5000 mg/kg [12].

Table 1
Dose-dependent mortality rate of mice in the acute toxicity study of AME extract

Dose (mg/kg)	1000	2000	2250	2500	3000	4000	6000
Number of mice per group	6	10	10	10	10	10	6
Number of deaths	0	3	4	6	7	8	6
Mortality rate	0	30	40	60	70	80	100

On the basis of this estimated LD_{50} value, two doses were selected for oral administration in the antitumor efficacy study: 200 mg/kg and 100 mg/kg, corresponding to approximately 1/10 and 1/20 of the LD_{50} , respectively. For this purpose, AME extract was dissolved in distilled water to prepare solutions at concentrations of 20 mg/mL and 10 mg/mL. A volume of 0.1 mL per 10 g of body weight was administered orally to each mouse. Additionally, the same solutions were applied topically to the shaved dorsal area of the mice to evaluate the effect of topical treatment.

3.2. Simulation of a melanoma model in Swiss *albino* mice

In the immunocompetent group, mouse mortality was dependent on the density of B16F10 cell inoculation. At the lowest concentration (5×10^4 cells per mouse), no mortality was observed, whereas the highest concentration resulted in approximately 50% mortality. The rate of tumor formation was proportional to the number of implanted cells in immunocompetent mice. The injection of 5×10^6 cells per mouse resulted in immediate tumor formation, with visible blackening at the injection site observed as early as one day post-injection. The injection of 5×10^5 cells or 5×10 cells per mouse led to tumor

development after a latency period ranging from 5–9 days (Table 2). Moreover, the resulting tumors in the immunocompetent group were soft and loosely textured upon palpation (Fig. 1).

Table 2
Survival and tumor latency according to B16F10 cell density in immunodeficient vs. immunocompetent mice

Group	Number of cell implantation	n	Survival rate (%)	Tumor latency (day) (Mean $\pm$ SD)
Immunocompetence	5×10^4	6	100	9 ± 2
	5×10^5	7	57.1	5 ± 4
	5×10^6	6	50.0	1 ± 0
Immunodeficiency	5×10^4	10	60.0	2 ± 1
	5×10^5	10	60.0	1 ± 0
	5×10^6	10	60.0	1 ± 0
Control	---	12	100.0	---

In the immunodeficient group induced with cyclophosphamide, the survival rate was only 60%. Most tumors formed as early as the day following injection and exhibited continuous growth. Upon palpation, the tumors were firm and solid in texture (Fig. 1). Moreover, the blackened area at the injection site of 5×10^6 cells was noticeably larger than those observed at the two lower cell densities. The tumor size in these three groups increased rapidly from Day 1 to Week 2. Consistently, all three groups exhibited a steady increase in tumor volume from Day 1, reaching its peak at Week 3, indicating active tumor proliferation. At this time point, the group injected with 5×10^5 cells showed the most aggressive growth ($\sim 350 \text{ mm}^3$), followed by the group injected with 5×10^4 cells ($\sim 300 \text{ mm}^3$) and the group injected with 5×10^6 cells ($\sim 250 \text{ mm}^3$). After Week 3, the tumor volume in all the groups began to decrease. From week 5 onward, signs of surface necrosis were observed. By the end of the experiment (week 8), the group injected with 5×10^5 cells retained the largest tumor volume (Fig. 2).

Histological examination of the tumors in the immunodeficient group confirmed the presence of malignant melanoma cells within the epidermal layer. The tumors were characterized by proliferating melanocytes with enlarged nuclei and cytoplasm containing abundant brown–black melanin granules (Fig. 3). Microvascular proliferation was observed in tumor tissues across all three experimental groups. In mice injected with 5×10^5 cells, the tumor displayed a nodular architecture, with tumor cells aggressively invading skeletal muscle and neural structures, reaching a maximum invasion depth of 3 mm. In the two remaining groups of mice, tumor cells infiltrated the dermal soft tissues, including adipose tissue and skeletal muscle, with a recorded invasion depth of 1.5 mm. Notably, no evidence of

vascular or perineural invasion was detected in the mice injected with 5×10^4 cells; however, such invasions were observed in the group injected with 5×10^6 cells.

Therefore, for the *in vivo* modeling of malignant melanoma in Swiss *albino individuals*, a protocol utilizing immunodeficient mice pretreated with cyclophosphamide, followed by the subcutaneous injection of B16F10 melanoma cells at a density of 5×10^5 cells in 0.1 ml of PBS, was selected to investigate the antimelanoma effects of AME.

3.3. Antimelanoma effects of *Annona muricata* leaf extract

After the 6-week experimental period, the combination of oral and topical treatments resulted in a survival rate (75.0%) comparable to that of the untreated group. In contrast, the oral administration group presented a greater survival rate, whereas the topical application group presented a lower survival rate (Table 3). In the untreated group, the tumor volume increased rapidly during the first three weeks (Fig. 4). Compared with the untreated group, the PO200 group also presented an increase in tumor volume, although to a lesser extent. Notably, high-concentration topical treatment (TO20) and combination therapy (P100 + T010) significantly inhibited tumor growth from Week 2 to Week 6.

Table 3
Survival rates of the AME-treated and untreated groups

Groups	n	Survival rate (%)
PO200	14	42.9
TO20	7	85.7
PO100 + T010	8	75.0
Untreated	8	75.0

At the end of the experiment, the tumors in the untreated group were markedly large with dark, well-defined margins (Fig. 5). Specifically, the tumors in the combination treatment group were the smallest and lightest in color among all the groups. Histological analysis revealed vascular invasion in 100% of the samples from the three AME-treated and untreated groups (Fig. 6). The PO200 group presented the lowest average Clark level (4.25 ± 0.5) and the shallowest invasion depth (1.0 ± 0.0 mm). In contrast, both remaining AME-treated groups reached Clark level V (Table 4). Moreover, the topical group had the smallest average horizontal tumor diameter (3.0 mm), which was significantly different from that of the untreated group. In the PO200 group, the dermis contained tumor tissue composed of proliferating melanocytes with large nuclei and abundant cytoplasm with melanin pigment. Furthermore, the tumor cells invaded the reticular dermis. Moreover, in the TO20 group, the dermis contained tumor tissue composed of proliferating melanocytes with large nuclei and abundant cytoplasm with melanin pigment. Additionally, these tumor cells were observed to invade the subcutaneous soft tissue. Similarly, in the combination group (P100 + T010), the dermis contained tumor tissue composed of proliferating

melanocytes with large nuclei and abundant cytoplasm with melanin pigment. Moreover, these tumor cells also invade the subcutaneous soft tissue. Finally, in the untreated group, the dermis contained tumor tissue composed of proliferating melanocytes with large nuclei and abundant cytoplasm with melanin pigment. Likewise, these tumor cells were found to invade the subcutaneous soft tissue (Fig. 6).

Table 4. Evaluation of the histological indices of the AME-treated groups compared with those of the untreated groups

Indices \ Groups	PO200	TO20	PO100+TO10	Untreated
Average Clark index	4.25 ± 0.5	5 ± 0	5 ± 0	5 ± 0
Average horizontal diameter (mm)	4.25 ± 0.65	3.0 ± 0	4 ± 0.82	4.75 ± 0.96
Average invasion depth (mm)	1 ± 0	1.375 ± 0.25	1.25 ± 0.29	1.375 ± 0.48

4. DISCUSSIONS

Annona muricata Linn is a tropical plant species recognized for its edible fruit, which exhibits significant medicinal properties alongside reported toxicological concerns. In our study, oral administration of *Annona muricata* leaf extract (AME) resulted in an estimated LD₅₀ of approximately 2180.28 ± 84.76 mg/kg, which is considered “slightly hazardous” according to the GHS classification. *In vivo* studies by De Sousa OV et al. (2010) demonstrated that oral administration of the ethanol extract of *Annona muricata* leaves resulted in an LD₅₀ of 1670 mg/kg (95% CI: 1240–2260 mg/kg) [15]. Similarly, Acesio NO et al. (2017) reported 100% mortality in all experimental groups within 24 hours following oral administration of a 96% ethanol extract of *Annona muricata* leaves at doses ranging from 1000 to 2000 mg/kg [16]. In contrast, an acute toxicity study by Siti Norliyana Zubaidi et al. (2023) indicated that the 80% ethanol extract of *Annona muricata* leaves could be safely administered at a dose of 2000 mg/kg [17]. Furthermore, Agu et al. (2017) reported a moderately hazardous LD₅₀ value of 1918.33 mg/kg for leaf and fruit extracts from methanol [18], which may be attributed to the use of methanol as the extraction solvent. The methanol extract demonstrated a distinct extraction efficiency compared to the ethanol extract and was associated with the co-extraction of a broader range of potentially toxic compounds, which may contribute to the increased toxicity observed.

The leaf extract of *Annona muricata* contains several bioactive constituents, primarily acetogenins, alkaloids, and flavonoids. Among these, acetogenins are the predominant compounds and are considered chiefly responsible for the acute toxicity of the leaf, particularly its neurotoxic effects [19]. Notably, several studies have demonstrated that acetogenins isolated from *Annona muricata* leaves exhibit cytotoxic activity against tumor cells [20–26], suggesting their potential therapeutic relevance. However, the presence of these compounds may also contribute to the observed toxic effects of ethanol-

based extracts. Therefore, the future development of ethanol extracts from *Annona muricata* leaves as therapeutic agents requires rigorous standardization of both the raw material and the extraction process to ensure consistent safety and efficacy. In our study, determining acute toxicity is a critical step in establishing the safety profile of AME and was used to define the therapeutic doses (100 and 200 mg/kg, equivalent to 1/20 and 1/10 of the LD₅₀, respectively) for evaluating its antimelanoma effects. Moreover, further investigations should include subchronic toxicity assessments to evaluate potential long-term cumulative effects, as well as detailed chemical characterization to elucidate the relationships between specific acetogenin contents and both toxicological and pharmacological outcomes.

Melanoma is recognized as the most dangerous type of skin cancer and is characterized by high mortality rates and a propensity for developing metastatic lesions that evade immune surveillance [27]. The B16F10 cell line, which is used extensively in melanoma research, represents the 10th subclone of the parental B16 cell line. Originally derived from C57BL/6 mice, this pigment-producing melanoma cell line has strong metastatic potential, with the ability to spread to organs such as the spleen, liver, and lungs [28, 29]. Although implantation of the B16F10 melanoma cell line in the C57BL/6 inbred mouse strain is widely employed in experimental oncology models because of its immunocompatibility and reproducibility [30, 31], maintenance and breeding of the C57BL/6 inbred mouse strain can present logistical and financial challenges in certain research settings. As a practical alternative, Swiss *albino* mice offer several advantages, including ease of handling, lower cost, and broader availability. When combined with an appropriate immunosuppression protocol, Swiss *albino* mice are capable of supporting B16F10 cell inoculation and can serve as a viable model for melanoma research, providing a more accessible and cost-effective platform for preclinical evaluation.

Our results revealed significant differences in tumor development between immunocompetent and immunodeficient Swiss *albino* mice. In the group that did not receive immune inhibitors, the tumors were soft and loosely textured upon palpation. In contrast, mice pretreated with cyclophosphamide developed solid tumors with firm consistency, indicating more aggressive growth characteristics. These findings are consistent with those of previous studies worldwide [32, 33] and suggest that immunosuppression plays a critical role in facilitating solid tumor formation and progression in this melanoma model. Melanoma tumors in the immunodeficient group were observed as early as one day post-injection and exhibited continuous growth throughout the study period. Interestingly, the growth dynamics varied depending on the number of cells injected. The group that received 5×10^6 cells presented the earliest tumor formation, whereas the group that received 5×10^5 cells presented the most aggressive growth, reaching approximately 350 mm³ by week 3. This was followed by the 5×10^4 cell group (approximately 300 mm³) and the 5×10^6 cell group (approximately 250 mm³). The relatively slower tumor growth observed in the group injected with 5×10^6 cells may be attributed to a 'cell overcrowding' effect. In a nutrient- and oxygen-limited microenvironment, excessive cell density can lead to competition for resources, thereby restricting tumor expansion [34, 35]. Rapidly growing tumors consume large amounts of oxygen and nutrients; if the vascular system does not develop in time to meet this demand, regions within the tumor may become necrotic and shrink. To compensate, tumors can stimulate angiogenesis

to restore the oxygen supply, enabling cancer cells to survive and regrow. In this study, necrosis was observed in both groups injected with 5×10^5 and 5×10^6 cells, likely due to rapid tumor growth and subsequent ischemia. In contrast, no necrosis was detected in the group injected with 5×10^4 cells, possibly due to the smaller tumor size and reduced metabolic demand [34, 36].

The present study provides preliminary yet valuable evidence of the antitumor efficacy of *Annona muricata* leaf extract (AME) against melanoma in a murine model. Both oral and topical routes of administration exhibited distinct therapeutic effects. Oral administration of AME in the PO200 group was associated with the lowest average Clark level (4.25 ± 0.5) and the shallowest invasion depth (1.0 ± 0.0 mm), suggesting that systemic delivery may play a pivotal role in suppressing vertical tumor invasion and limiting deep tissue penetration. In contrast, high-concentration topical treatment in the TO20 group significantly restricted horizontal tumor expansion, resulting in the smallest average tumor diameter (3.0 mm), highlighting its efficacy in controlling the local tumor burden. Compared with the other groups, the combination group (P100 + TO10) presented the greatest overall therapeutic effect, with consistent tumor volume suppression from Week 2 to Week 6 and visibly smaller, lighter tumors. These findings highlight the complementary benefits of dual-route administration: oral delivery enables the systemic distribution of bioactive compounds to target disseminated tumor cells, whereas topical application ensures high local concentrations at the tumor site to inhibit surface-level proliferation. This synergistic approach aligns with current evidence supporting multimodal delivery strategies in cancer therapy, which often outperform monotherapies by addressing both systemic and localized disease components [37, 38].

Despite these promising antitumor effects observed with AME, several limitations remain. Histological analyses revealed persistent vascular invasion and subcutaneous infiltration across all treatment groups, indicating that tumor progression was not completely suppressed. Additionally, the study employed only a single dose for each administration route, which precludes a comprehensive evaluation of dose–response relationships and optimal therapeutic windows. The absence of biochemical and molecular data further limits mechanistic interpretation, making distinguishing between the systemic and localized effects of AME difficult. These limitations warrant careful consideration and underscore important directions for future research. First, dose-escalation experiments are essential for determining both the minimum effective dose and the maximum tolerated dose of AME. Second, biochemical and immunohistochemical analyses should be conducted to elucidate the molecular mechanisms underlying its antitumor effects. Third, long-term safety assessments, including subchronic toxicity and recurrence monitoring, are necessary to assess the sustainability and potential risks of prolonged use. Moreover, future investigations should explore combination strategies involving AME and established therapies, such as immune checkpoint inhibitors or BRAF/MEK inhibitors, to evaluate potential synergistic effects and broaden its clinical applicability in melanoma treatment. Integrative therapeutic approaches may facilitate the positioning of *Annona muricata* leaf extract as an adjuvant agent in melanoma management, thereby bridging traditional herbal medicine with contemporary oncological practice.

5. CONCLUSION

Annona muricata leaf extract demonstrated a favorable safety profile (LD₅₀: 2180.28 ± 84.76 mg/kg). In a B16F10 melanoma mouse model in immunodeficient Swiss *albino* mice, oral administration or topical application, especially combination treatment, significantly reduced the tumor burden and inhibited tumor progression. These findings support its potential as a promising adjuvant therapy for melanoma.

Declarations

Ethics approval and consent to participate

All experimental protocols were approved by the Committee on Animal Research and Ethics of the University of Medicine and Pharmacy at Ho Chi Minh City under Decision No. 3674/GCN-HĐĐDNCTĐV, dated 20/11/2024.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare no competing interest.

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Authors' contributions

Conceived and designed experiments: NT-H, HP-D and HTV-N. Performed experiments: HN-N, BH-N, VK-L and HTV-N. Analyzed data: NT-H and HTV-N. Manuscript drafting and revision: NT-H, HP-D and HTV-N. All authors read and agreed to the final manuscript.

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Clinical trial number

Not applicable.

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Figures

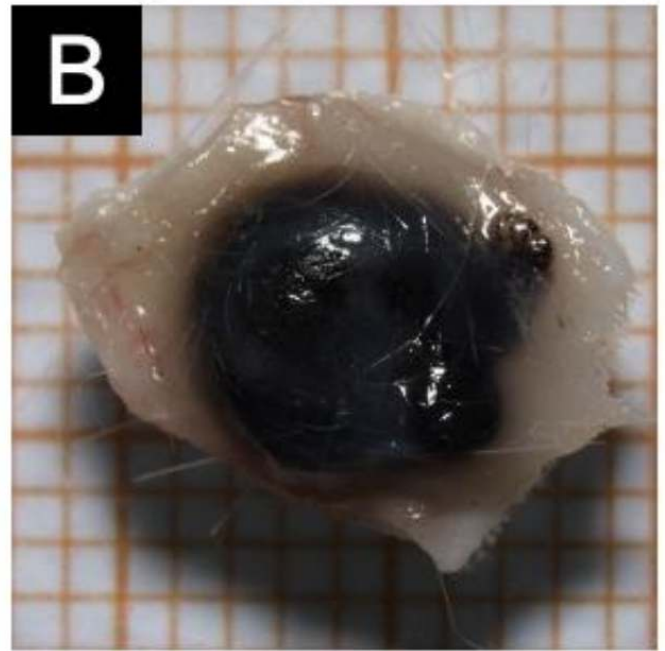
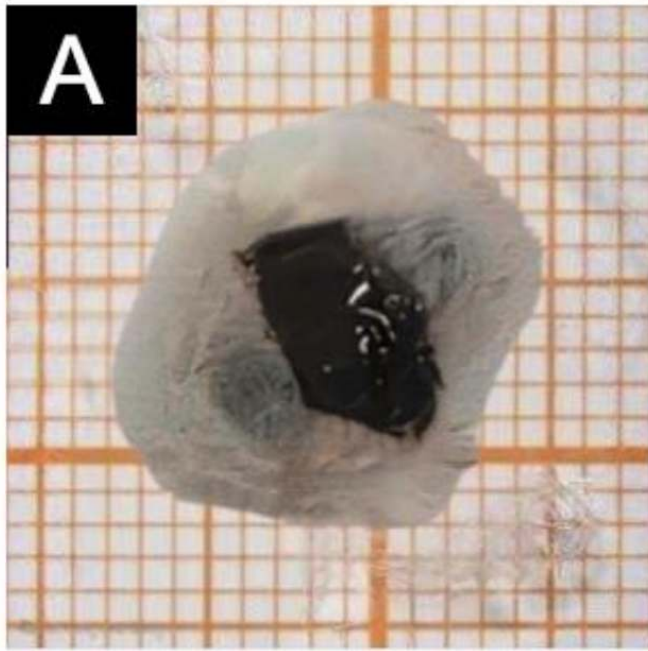


Figure 1

Macroscopic images of tumors from immunocompetent (A) and immunodeficient (B) mice under a stereomicroscope after surgery at the end of the experiment.

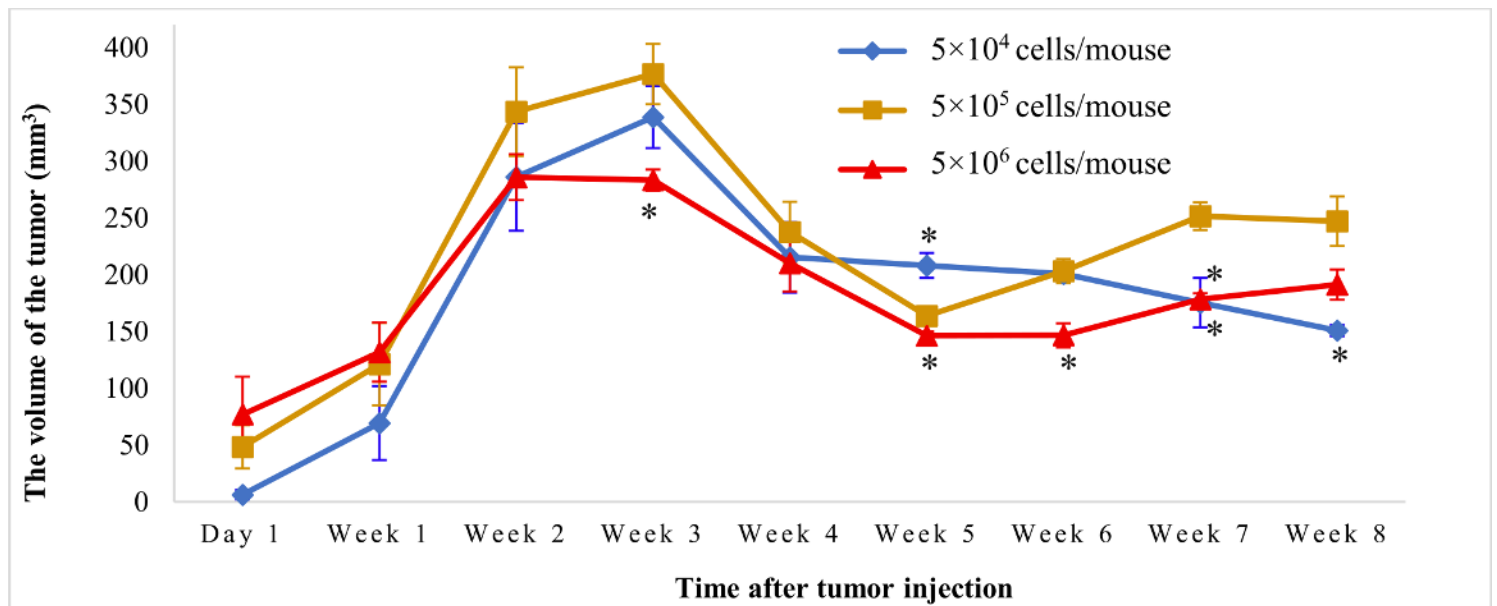


Figure 2

Tumor volume (mm³) by B16F10 cell density in the immunodeficient group

*Note: * = $p < 0.05$ vs 5×10^5 cells/mouse*

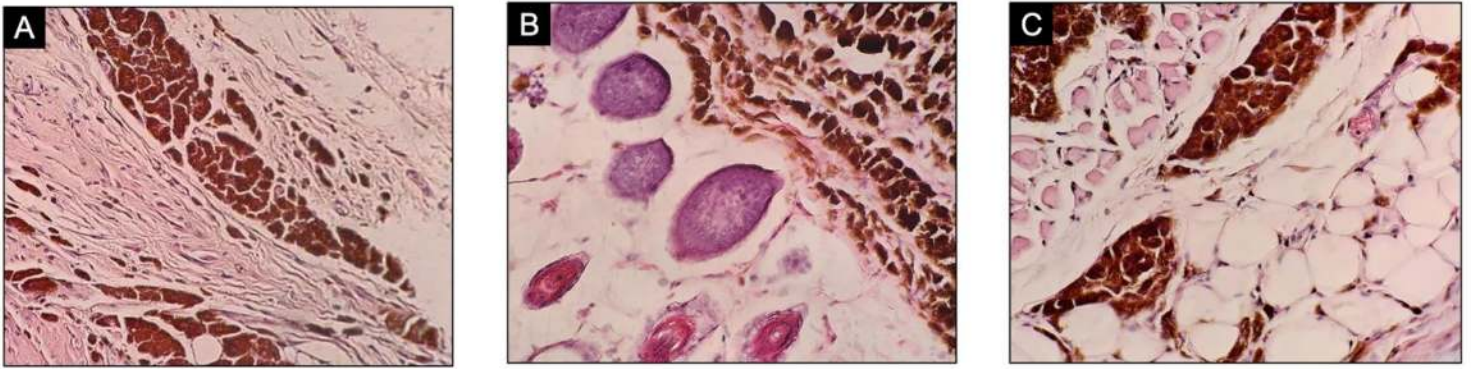


Figure 3

Histological images of tumor sections under 40X objective magnification

(A) 5×10^4 cells/mouse, (B) 5×10^5 cells/mouse, (C) 5×10^6 cells/mouse

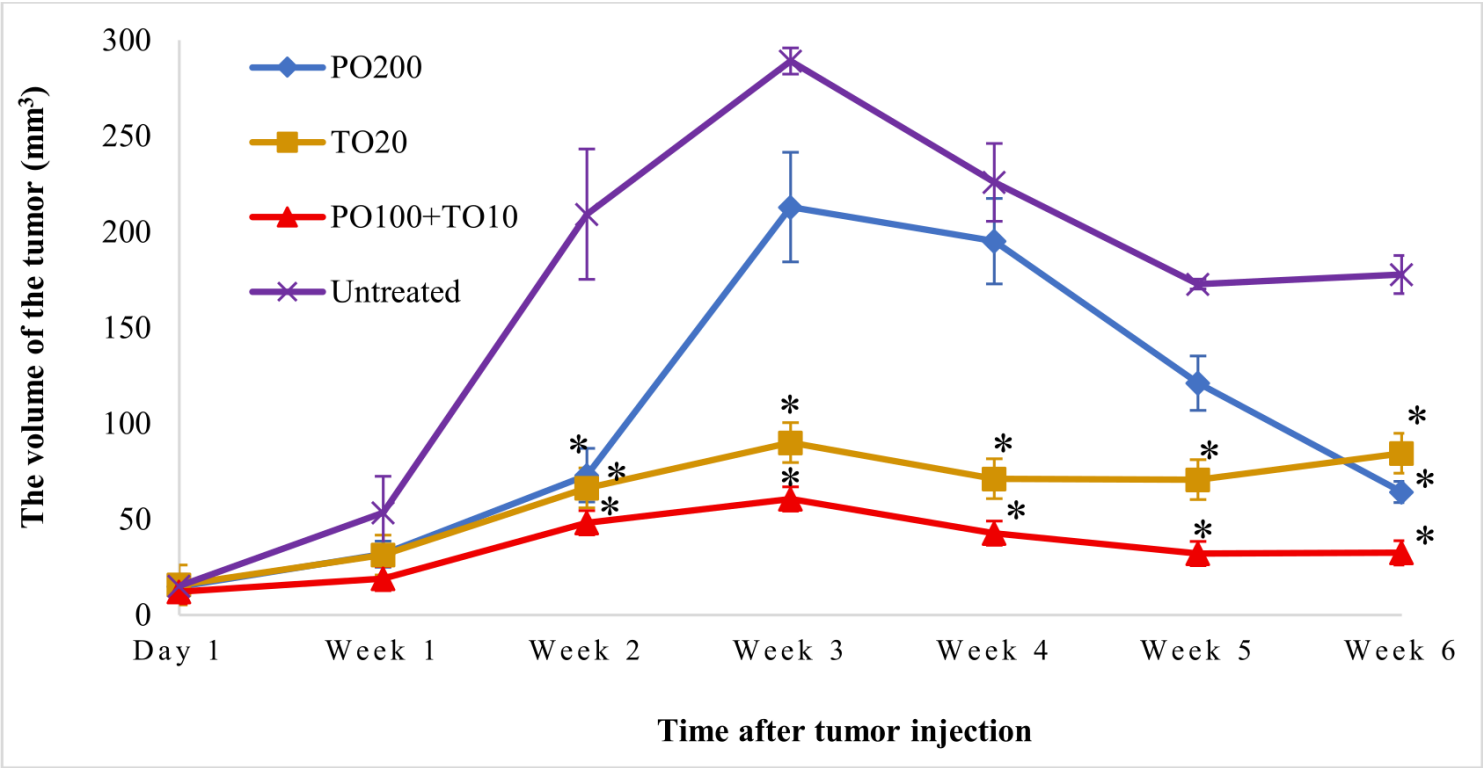


Figure 4

Tumor volume (mm³) in the AME-treated and untreated groups

Note: * = $p < 0.05$ vs untreated

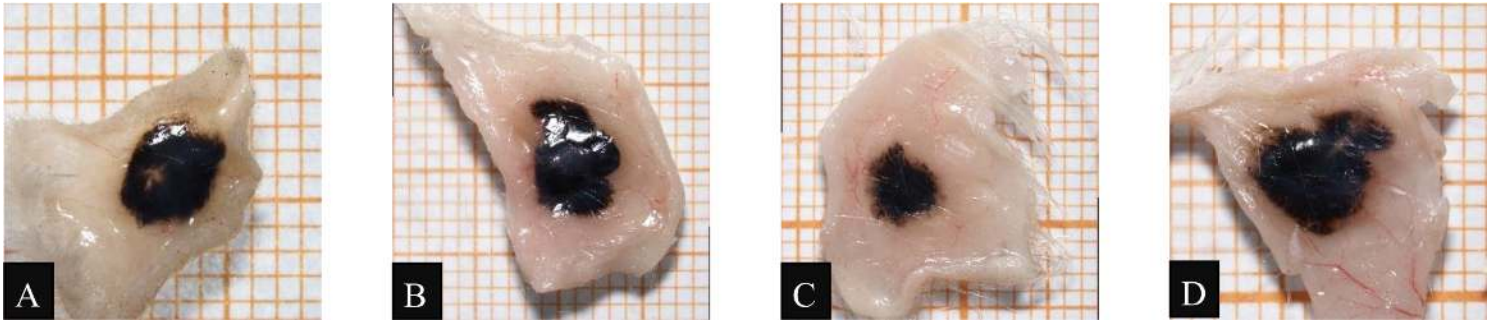


Figure 5

Macroscopic tumor features of AME-treated mice after oral administration (A), topical application (B) or combination (C) and the untreated group (D)

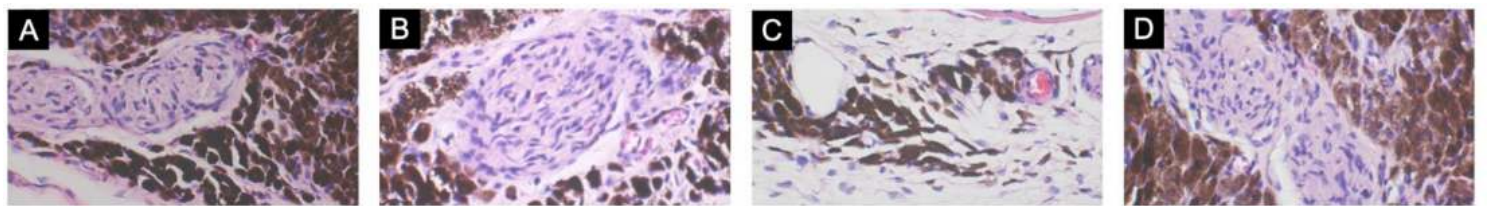


Figure 6

Histological images of tumor sections under 40X objective magnification

(A) PO200; (B) TO20; (C) PO100+TO10; (D) Untreated